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GIANT CANADIAN SNAKES AND FORENSIC PHYLOGENETICS

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ABSTRACT: Phylogenetics has been shown to be a useful forensics tool, and this use can be expanded to the conservation arena. Here we employ phylogenetics to test a cryptozoological specimen for species identification. Over the past decade, one of us (JW) has been collecting and recording stories about unusual animals in the Manitoba wilderness of Canada. Recently, a two meter plus shed was found in the wilderness of Manitoba, Canada. We isolated DNA from the shed and amplified and sequenced a portion of the cyt-b gene. This sequence was compared in a phylogenetic context with cyt-b sequences from a number of snake taxa. Based on these analyses, the shed appears to have come from a *Boa constrictor* from northeastern South America.

Key Words: Giant Canadian snakes, forensics, phylogenetics, *Boa constrictor*, cytochrome b, conservation

INTRODUCTION

Molecular phylogenetics and forensics

Molecular phylogenetics has become an entrenched approach to hypothesize relationships among species. As the science has developed, applications have expanded. One relatively new area of study is wildlife forensics. The U.S. Fish and Wildlife's National Fish and Wildlife Forensics Laboratory uses traditional and molecular forensics techniques to identify to the species, subspecies, and even individual level a variety of samples including bones, tusks, hides, leather goods and more. This and similar labs can identify body parts belonging to endangered species, determine how many different individuals a particular sample contains, or determine whether the blood on a hunter's jacket comes from a poached animal. The ready availability of DNA sequences and microsatellite data for a wide variety of species facilitates the identification of unknown samples.

Conservation, cryptozoology, and molecular phylogenetics

Stories of undocumented, unusual or relict animals abound, from the familiar Loch Ness monster, Bigfoot (Sasquatch) and Yeti, to the less familiar megalania (a giant Australian monitor lizard) and the ferocious African Nandi bear. Evidence for such legendary beasts generally consists of personal accounts of sightings, unusual footprints, and blurry photographs. Concrete physical evidence is rarely available to support such sightings. In rare instances, hair found in the vicinity of Sasquatch sightings has been tested, but these hairs have inevitably turned out to belong to some known mammal such as an elk or a bear. Milinkovitch et al. (2004) employed satire in an April Fools paper on the molecular phylogenetics of yeti and primates. Although farcical, such a use of phylogenetics as a tool is completely realistic. For example, Metzker et al. (2002) used phylogenetics as a forensic tool to track down HIV transmission in a criminal case and Giao et al. (1998) presented molecular phylogenetic evidence for the existence of two undescribed species of muntjac in Vietnam.

Given that resources for conserving rare and endangered organisms are extremely limited, it is critical to be able to identify real conservation priorities to ensure efficient and proper allocation of those resources. When species novel to science are discoverable via any indirect method, such as molecular phylogenetics, then that method should be considered a tool of conservation. For example, if an undescribed species of forest ungulate in the Pacific northwest of North America was discovered by molecular phylogenetic analysis of hair, would not the conservation approach to that region change in some way?

Over the past decade, one of us (JW) has been collecting and recording stories about unusual animals in the Manitoba wilderness of Canada. Many of the creatures described by Native peoples are highly peculiar, none less so than a group of large "stove pipe" sized snakes. Encounters with such animals have been described to

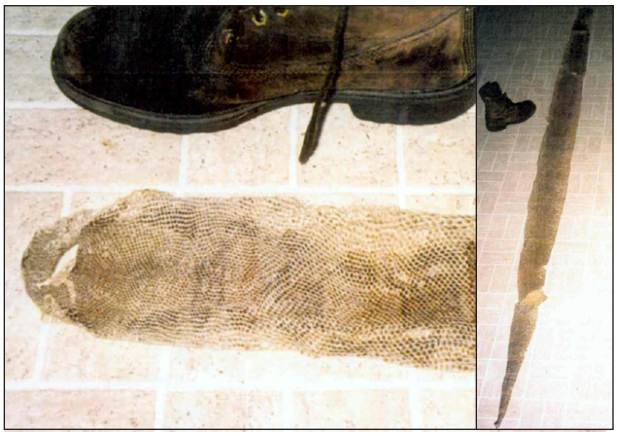

Figure 1. Photographs of the shed skin from an unidentified species of snake collected in Manitoba, Canada. The shed was approximately 2.6m in total length.

JW numerous times by both Native and non-Native people, although always with some reticence. In general, biologists would consider the probability of existence of such a large ectotherm in far northern latitudes as remote (Froom 1972; Preston 1982). Nonetheless, we were intrigued when JW asked if we (BIC, MEW) would be interested in looking at the DNA from a large shed skin found in central Manitoba.

The goal of this study was simply to try identify the taxon of the shed skin and in the process test the hypothesis that the shed was from a previously unknown species of snake from the northern latitudes of North America.

METHODS

A shed skin from a large individual (>2m; Fig. 1) was collected ca. 100 meters from the shoreline of northern Lake Martin in southern Manitoba. The shed was found in a crotch of a tree near the ground. DNA was isolated from the ventral scutes. Following standard procedures, as described below, cytochrome b was amplified and sequenced. Cyt-b was employed because sequences are readily available for numerous snake taxa.

The cytochrome b gene was amplified using Advantage cDNA PCR kits without GC melt (Clontech Laboratories) and with universal primers (Burbrink et al. 2000).

Cytochrome b primers:

L14910: GACCTGTGATMTGAAAACCAYCGTTGT

H16064: CTTTGGTTTACAAGAACAATGCTTTA

PCR amplification was conducted on a Perkin-Elmer thermocycler. Negative controls were used to check for contamination of experimental samples. PCR amplification cycles were 7 min. @ 94°C, 30 s @ 94°C, 30 s @ 46°C for annealing, 1 min @ 72°C, 30-40 cycles. PCR amplifications were visualized and tested for correct fragment sizes on a 1% agarose gel using a 1KB ladder (Gibco).

The PCR products were ligated into TOPOTA vectors and cloned using the TOPOTA cloning kit (Invitrogen). The products were transformed into TOP 10 competent *E. coli* cells and grown on LB plates (Appendix 7) at 37°C for 12+ hours. X-Gal was applied to the plates for proper colony selection. Colonies were picked from plates and grown in LB with 50µg/ml kanamyacin for more than 14 hours. Plasmid DNA was isolated from the competent *E. coli* cells using PerfectPrep Plasmid DNA kits (Eppendorf 5 Prime), cut with EcoRI restriction enzymes, and tested on a 1% agarose gel to verify the plasmid contained the desired insert. DNA was sequenced using the Sequitherm Excel II DNA Sequencing Kits (Epicentre Technologies) with M13-Forward and M13-Reverse primers. Advantage-2 DNA polymerase was substituted for the Sequitherm Excel kit polymerase to improve sequencing length and reading efficiency. The sequencing reactions were performed using cycle sequencing conditions and loaded on a Licor 4000L for automated sequencing.

Analytical Strategy

Sequences from a broad array of snakes were downloaded from Genbank (Table 1), aligned (using Sequencer and by eye) with the sequence from the Manitoba shed and analyzed within a parsimony framework using PAUP* (Swofford 1998) and TNT (Goloboff et al. 2000). The characters were treated as equally weighted and included all positions. Clades were evaluated by bootstrap proportions (Felsenstein 1985), decay indices (Bremer 1994), and character support.

We employed a two step phylogenetic approach to get as close as possible to identifying the specimen. First, we included the Manitoba sequence in an analysis that

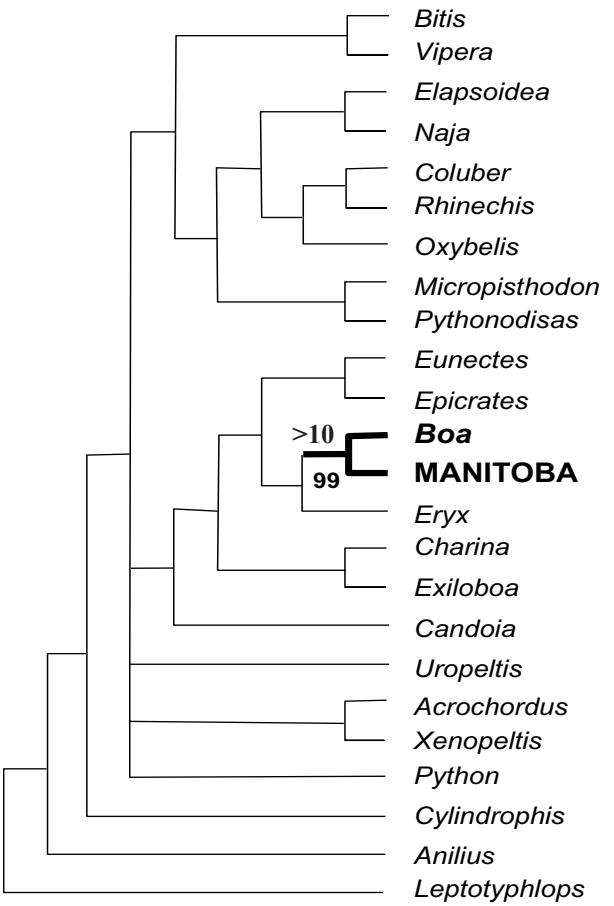

Figure 2. The inferred phylogenetic position of the Manitoba shed when compared across snake phylogeny. Only the node of interest, the location of the Manitoba specimen, is described by bootstrap proportion and decay index. As noted in the text, those values are the highest for any node. The descriptive statistics of the tree overall were tree length = 3005 steps, CI = 0.37, and RI = 0.34. See Table 1 for the Standard English names of the snake genera.

included taxa covering the major groups of snakes to determine its general relationships. Second, based on these results, we conducted a more detailed analysis to try pinpoint the specimen's identity and if possible the geographic origin of the snake. If the analyses did not infer a strong affinity for a known taxon, we could conclude that the specimen may be from an undescribed species.

RESULTS

Over 800bp of cyt-b was sequenced from the Manitoba specimen. In the initial analysis, three equally most parsimonious trees were inferred. The strict consensus tree depicted the Manitoba specimen to be most closely related to *Boa constrictor* (Fig. 2). That sister relationship exhibited a 99% bootstrap proportion and a decay index of >10. These values were the highest for any set of relationships in the initial analysis. Based on this result, we created a new data set that included sequences of *Boa constrictor* from seven localities across its range (Fig. 3) plus sequences from *Eunectes*, *Epicrates*, *Charina*, and *Python* (see Table 1). The aligned data set for these booids was 1127 positions. However, a number of the downloaded sequences were short (370 bases) so to avoid the inclusion of an extreme number of missing characters, the discussed analysis and descriptive statistics below were with the aligned 370 bases. It is worth noting that the inclusion of all the data resulted in the same set of relationships, except that the Suriname, French Guiana, Guyana, and Manitoba OTUs formed a polytomy. In the short data set, French Guiana is pulled

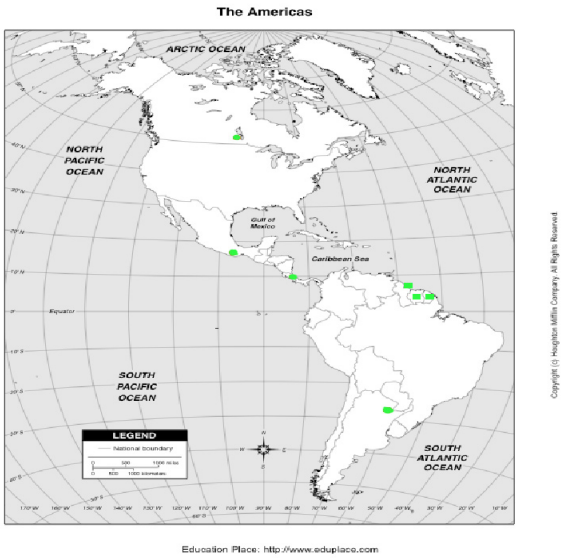

Figure 3. Localities of *Boa constrictor* samples. Not shown is the "Mexico" locality. The "*occidentalis*" locality is simply shown in the general area of the subspecies distribution. The green dots depict the localities of the specimens to which the Manitoba specimen is most closely related.

out of the polytomy. The subsequent analysis of just booids, including the specimens of *Boa constrictor* from seven localities, yielded two most parsimonious trees with a tree length = 262 steps, CI = 0.69, RI = 0.63, RC = 0.44 (Kluge and Farris 1969; Farris 1989). The strict consensus of those two trees inferred the Manitoba shed to be related to boas from northeastern South America (Fig. 4). The clade composed of specimens of *Boa constrictor* from northeastern South America had a bootstrap proportion of 94%, a decay index value of 6, and was diagnosed by eight characters. See Figure 4 for other details of the tree.

DISCUSSION

The two step phylogenetic approach provides strong evidence that the shed skin from the Manitoba specimen is not from an undescribed species. The initial analysis pointed to the specimen as related to *Boa constrictor* and from the more detailed analysis we were able to conclude that the shed was actually from a *Boa constrictor* and apparently one with ancestry in northeastern South America. We doubt that the animal successfully over-wintered in Manitoba, but clearly it did well enough to enter ecdysis. We also doubt that the shed was taken from a terrarium animal and placed in the tree because of the remote location of its discovery, although this is impossible to conclusively prove.

We recognize that there can be problems of extrapolating mtDNA results to nuclear genetic relationships (e.g. Taggart et al. 2001); however, given the broad geographic sample employed in this study we do not feel this is an issue. In fact, we expect that a test of our phylogenetic hypothesis with nuclear DNA would result in the same general conclusion.

Molecular phylogenetics allows definitive tests on purported cryptozoological specimens. While such analyses cannot dispute the existence of legendary beasts, it can shed light on individual claims. Native American lore has long hinted at the existence of large terrestrial snakes in Manitoba, Canada, but numerous "sightings" have not been confirmed. The discovery of a large snakeskin in a tree in Manitoba re-ignited such speculations. Our analysis of DNA isolated from the shed indicates that rather than coming from an undescribed Canadian

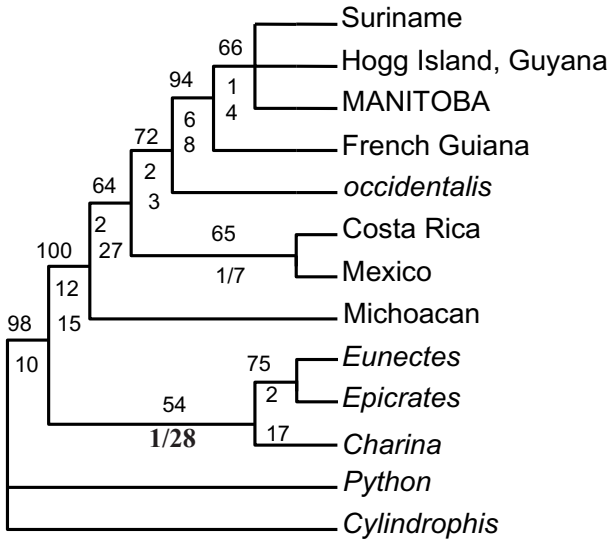

Figure 4. Strict consensus tree of booid analysis from the two most parsimonious trees. See text for analytical details and other descriptive statistics. Values above the branches are bootstrap proportions; below the branches the top number is the decay index and the bottom number is the total character support. All locality OTUs are *Boa constrictor*. The locality of the sequence for the OTU "*occidentalis*" was not given, only the subspecific epithet, which is from Argentina-Paraguay. The OTU "Mexico" is as precise as given in the reference. See Table 1 for the Standard English names of the snake genera.

species, the skin was shed by an individual probably from the pet trade, a *Boa constrictor* from northeastern South America. While there is documentation of alien booid populations in North America (e.g. Snow et al. 2007), no established populations are known from the northern latitudes of the continent and none are expected. Does Manitoba have its own species of giant snake? We don't know. The stories from the Native peoples suggest something out of the ordinary exists. But the specimen in question is not evidence for its existence.

ACKNOWLEDGMENTS

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Table 1. Accession numbers and localities, as given in the accession account, for the sequences used in the study.

Acrochordus granulatus (Little Filesnake) AF218741; *Anilius scytale* (False Coralsnake) U69738; *Bitis nasicornis* (Rhinoceros Viper) AF471068; *Boa constrictor* (Boa Constrictor) A4575035 – Michoacan, Mexico, U69750 ¬– "collected in Mexico", U69749 – Costa Rica, U69748 – Hogg Island (Guyana), U69745 – *B. c. occidentalis* (no locality), U69743 – Suriname, AF039267 – French Guiana, EU004913 – Manitoba, Canada; *Candoia carinata* (Pacific Ground Boa) AY099984; *Charina trivirgata* (Rosy Boa) AY099988; *Coluber zebrinus* (Zebra Racer) AY188043; *Cylindrophis rufus* (Pipe Snake) AF471032; *Elapsoidea sundevallii* (Venomous Gartersnake) AY188016; *Epicrates striatus* (Haitian Boa) U69799; *Eryx johnii* (Sand Boa) AY099987; *Eunectes murinus* (Green Anaconda) U69808; *Exiliboa placata* (Oaxacan Dwarf Boa) AY099989; *Leptotyphlops columbi* (San Salvador Threadsnake) AF544671; *Leptotyphlops humilis* (Western Threadsnake) AY099991; *Micropisthodon ochraceus* (Flat-headed Snake) AY188030; *Naja nubiae* (Nubian Spitting Cobra) AF399752; *Oxybelis aeneus* (Brown Vinesnake) AF471056; *Python molurus* (Indian Python) AY014890; *Python regius* (Ball Python) AF337116; *Python reticulatus* (Reticulated Python) AY014896; *Pythonodipsas carinata* (Western Keeled Sanke) AY188036; *Rhinechis scalaris* (Ladder Snake) AY486932; *Vipera ammodytes* (Long-nosed Viper) AY311380; *Uropeltis phillipsi* (Phillips Indian Earthsnake) AF471034; *Xenopeltis unicolor* (Sunbeam snake) AF544668.

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